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Responses of invertebrates to temperature and water stress: A polar perspective

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Abstract

As small bodied poikilothermic ectotherms, invertebrates, more so than any other animal group, are susceptible to extremes of temperature and low water availability. In few places is this more apparent than in the Arctic and Antarctic, where low temperatures predominate and water is unusable during winter and unavailable for parts of summer. Polar terrestrial invertebrates express a suite of physiological, biochemical and genomic features in response to these stressors. However, the situation is not as simple as responding to each stressor in isolation, as they are often faced in combination. We consider how polar terrestrial invertebrates manage this scenario in light of their physiology and ecology. Climate change is also leading to warmer summers in parts of the polar regions, concomitantly increasing the potential for drought. The interaction between high temperature and low water availability, and the invertebrates' response to them, are therefore also explored.

Keywords: Cross tolerance; rapid cold hardening; anhydrobiosis; cryoprotective dehydration; sub-lethal characteristics; climate warming

1. Introduction

1.1. The trials of being an invertebrate

Invertebrates, more so than any other animal group, are at the whim of their environment. Unlike birds and mammals, which are able to regulate their internal body temperature, invertebrates are poikilothermic ectotherms and their body temperature is highly influenced by, and varies markedly with, the environmental temperature (Speight *et al.* 2008). While cold-blooded vertebrates, such as fish, reptiles and amphibians, are also poikilothermic ectotherms, they are not generally as diminutive

as invertebrates. Even the smallest vertebrate recorded, the Papua New Guinea frog *Paedophryne amanuensis* (7.7 mm in length), dwarfs the vast majority of invertebrates (Rittmeyer *et al.* 2012). Cold-blooded vertebrates accordingly have a smaller surface area to volume ratio than invertebrates and therefore have more time to respond to changes in temperature. This means that invertebrates are more susceptible to injuries following either rapid cooling (Czajka 1990) or warming (Chidawanyika and Terblanche 2011). A small body size also means invertebrates are generally more vulnerable to desiccation than their larger-bodied vertebrate relatives.

1.2. Polar climate

In few places are invertebrates more directly impacted by their environment than in the Arctic tundra (Strathdee and Bale 1998) or the fellfields of the Antarctic (Block *et al.* 2009; Hogg *et al.* 2006). Air temperatures regularly fall below -10^{o}C during the winter in the maritime Antarctic and, in regions such as the continental Antarctic and High Arctic, frequently drop below -40^{o}C (Block *et al.* 2009; Convey 2013; Sformo *et al.* 2010; Strathdee and Bale 1998). Invertebrates buffer these temperatures behaviourally to some extent (Hayward *et al.*, 2003) by moving beneath the snow, within the soil profile, or into cryptogams like mosses, lichen and algae (Bengtson *et al.* 1974; Burn 1986; Convey 1996; Convey and Smith 1997; Spaull 1973). However, even within these microhabitats, they can still be subjected to sub-zero temperatures on a daily basis throughout the winter (Davey *et al.* 1990; Block *et al.* 2009; Strathdee and Bale 1998). Microhabitat temperatures during the summer are also very low and rarely rise above 5^{o}C in the maritime and continental Antarctic, and slightly higher in the High Arctic (Block *et al.* 2009; Coulson *et al.* 1993; Strathdee and Bale 1998). The availability of liquid water also presents an important challenge. During the winter, water is locked up as snow and ice where it is inaccessible (Block *et al.* 2009) while, in summer, streams, lakes and rock pools, which form from melted ice and snow in spring, evaporate, resulting in drought (Convey *et al.* 2003). Again, behavioural responses can help reduce desiccation stress (Hayward *et al.*, 2000, 2001). However, because access to moisture is so restricted in both space and time at polar latitudes, physiological responses play a dominant role in determining species survival.

1.3. Overview

In response to low temperatures and water stress, polar terrestrial invertebrates express a suite of responses and strategies. However, these two stressors are often faced concurrently and the level of crossover between the strategies employed in response is considerable. A further interaction that may be faced currently, and will likely occur more frequently in the future, is that between high temperature and low water availability. Climate change is resulting in higher temperatures in summer and throughout the year in some polar regions (Arctic Council, 2005; Convey *et al.* 2009; Turner *et al.* 2009), increasing the potential for summer drought. The manner in which the resident invertebrate fauna, and potential colonisers, are able to tolerate and respond to this combination of stressors is therefore also pertinent.

It is important to note that the adaptations shown by polar terrestrial invertebrates are not necessarily uniquely different from non-polar species, simply that their adaptations are, in some cases, more developed because of the more extreme conditions they experience (Convey 1996). Studies on non-polar invertebrates are therefore also highly informative, and throughout this review these will be used to complement and expand on the concepts introduced for their polar counterparts. Further, there are certain stress tolerance strategies that are potentially relevant to polar systems that have only been described in non-polar invertebrates to date.

2. Responses to low temperature

Invertebrates that live in the polar regions can be at constant risk of their body fluids freezing and any associated injury (Mazur 1977). This risk is generally ameliorated by adoption one of two strategies - freeze-tolerance (= tolerance of internal ice formation) or freeze-avoidance (= avoidance of internal ice formation) (Bale 2002; Cannon and Block 1988; Convey 1996; Storey and Storey 1988; Zachariassen 1985).

2.1. Freeze-tolerance

Various polar invertebrates have been shown to use this strategy, including Diptera (e.g. *Belgica antarctica* [Benoit *et al.* 2009a], *Eretmoptera murphyi* [Worland 2010] and *Heleomyza borealis* [Worland *et al.* 2000]), Lepidoptera (e.g. *Gynaephora groenlandica* [Strathdee and Bale 1998]), Coleoptera (e.g. *Hydromedion sparsutum* and *Perimylops antarcticus* [Worland and Block 1999]) and nematoda (e.g. *Eudorylaimus coniceps* [Convey and Worland 2000]). While the continental Antarctic nematode, *Panagrolaimus davidi* (Wharton and Ferns 1995), has been shown to survive intracellular ice formation, perhaps indicative of a more general ability within polar nematodes, this form of injury is thought to be lethal to most other invertebrates (Block 1990). The vast majority of freeze-tolerant invertebrates therefore restrict ice formation to extracellular compartments. Key to this process is the accumulation of ice nucleating agents (INAs), such as specialised proteins (Block *et al.* 1990), food particles, crystalloid compounds (Lee *et al.* 1996) and microorganisms (Klok and Chown 1997; Worland and Block 1999), which act as heterogeneous surfaces for the promotion of water molecule aggregation (Bale 2002). By accumulating these agents in the haemolymph and gut, as well as in other tissues (Izumi *et al.* 2009), ice formation (which occurs at the supercooling point or SCP) is encouraged to take place extracellularly at high sub-zero temperatures (-3 to -10^{o}C) (Duman and Horwath 1983; Worland *et al.* 1992, 1993; Worland and Block 1999). At these temperatures, ice crystal growth is relatively slow, allowing water to move from the cytoplasm of cells and join the newly formed ice crystals. The cytoplasm therefore becomes more concentrated and the cell less susceptible to lysis via intracellular freezing (Worland and Block 1999). It should be noted that some invertebrates require an external trigger to survive internal ice formation. In the case of the wood centipede, *Lithobius forficatus*, inoculative freezing occurs at approximately -1°C and is essential for subsequent survival in the freeze-tolerant state (Tursman *et al.* 1994). Other invertebrates that require or may require inoculative freezing include nematodes and the midge, *B. antarctica* (Convey and Worland 2000; Elnitsky *et al.* 2008; Wharton 2003a, 2003b, 2011b).

However, freeze-tolerant invertebrates are still at risk from any one ice crystal in the extracellular space becoming too large and puncturing cells from the outside. They therefore also produce antifreeze proteins (AFPs) and/or antifreeze glycolipids (AFGLs). AFPs and AFGLs arrest the expansion of large crystals and instead promote the growth of many small crystals in a process called ice recrystallisation inhibition (Duman *et al.* 2004). AFGLs may also stabilise membranes and prevent the propagation of ice into the cytosol, and slow the growth of extracellular ice, reducing the rate of water flux and solutes across the cellular membrane (Walters *et al.* 2011). Even with the help of AFPs and AFGLs, ice formation is still able to distort proteins, membranes and other structures. Freeze-tolerant invertebrates thus accumulate polyhydric alcohols and sugars, such as glycerol, sorbitol and trehalose. Intracellularly, these cryoprotectants stabilise proteins and membranes, and prevent freezing, while extracellularly their function is to limit the osmotic imbalance that occurs during freezing, by maintaining water content above the "critical minimum cell volume" (Calderon *et al.* 2009; Holmstrup *et al.* 1999; Montiel 1998). Polyols and sugars also provide other benefits and aid metabolism.

127 *2.2. Freeze-avoidance*

128 In contrast to freeze-tolerant species, invertebrates which are freeze-avoiding are unable to withstand
129 any internal ice formation (Bale 1996; Cannon and Block 1988; Storey and Storey 1988; Zachariassen
130 1985). While seemingly disadvantageous in an environment which experiences temperatures close to
131 an invertebrate's SCP, these invertebrates avoid the dangers of both extracellular ice formation and
132 subsequent cellular dehydration that occur in freeze-tolerant species. Freeze-avoiding invertebrates
133 range from Alaska (e.g. the red flat bark beetle, *Cucujus clavipes puniceus* [Sformo *et al.* 2010]) and
134 the High Arctic (e.g. the mite, *Diapterobates notatus* [Coulson *et al.* 1995]) to the Antarctic continent
135 (e.g. *Cryptopygus antarcticus* [Block and Worland 2001; Cannon and Block 1988]), and outnumber
136 freeze-tolerant species in almost all cases. Freeze avoiding invertebrates can be separated into several
137 different categories to better define them ecologically and physiologically. These include, for
138 instance, true freeze-avoiding (lower lethal temperature [LLT] = SCP), chill tolerant (show minimal
139 pre-freeze mortality), chill susceptible (die well above their SCPs) and opportunistic survival (unable
140 to survive below their developmental threshold) (see Bale 1993). The SCP can also vary greatly
141 between and within species, and such classifications can thus become misleading. For example, the
142 summer-acclimatised polar collembolan, *Megaphorura arctica*, is classified as true freeze-avoiding or
143 chill tolerant, while the temperate aphid *Myzus persicae* is classified as chill susceptible. The reader
144 may therefore infer that summer acclimatised *M. arctica* is more cold tolerant. However, *M. arctica* in
145 summer has a high SCP of only -6^{o}C (Worland 1996), which is higher than the LLT of *M. persicae*
146 (Clough *et al.* 1990).

147 Mechanistically, freeze-avoidance revolves around a process termed supercooling - the prevention of
148 internal ice formation below the environmental freezing point. Enhanced supercooling is principally
149 achieved via three processes, and thus involves fewer core stress response mechanisms than freeze
150 tolerance (Bale 2002). The first is the removal of INAs and has been shown to lower the SCP by up to
151 20^{o}C in some invertebrates (Zachariassen *et al.* 1980; Burns *et al.* 2010). INAs are often removed by
152 moulting or ecdysis, the removal of the outer layer of the body and gut contents, which is a necessary
153 stage in the somatic development of arthropods (Hawes *et al.* 2007). Recent studies have shown that
154 moulting is highly dependent on temperature. For example, both the proportion of *C. antarcticus*
155 moulting at any one time (Worland and Convey, 2008) and the expression of moult-associated genes
156 (Burns *et al.* 2010) increase as temperatures fall, suggesting that the timing of moulting is an
157 important adaptive developmental trait. Starvation (Cannon and Block 1988; Sømme and Block 1982)
158 and food selection (Bokhorst *et al.* 2007; Worland and Lukešová 2000) may also be adaptive
159 processes which aid INA removal. While these processes help to rid INAs from the gut, or on the
160 cuticle, those in the rest of the body remain largely unaffected. To arrest ice nucleation here, as well
161 as any remaining INAs in the gut, freeze-avoiding invertebrates initiate a second element of the
162 supercooling process - the accumulation of AFPs. Through a non-colligative mechanism (thermal
163 hysteresis) of adsorption onto, and consequently inhibition of, embryonic ice crystals or INAs (Clark
164 and Worland 2008; Davies and Sykes 1997), AFPs reduce an organism's SCP relative to its melting
165 point (MP) (Bale 2002). Thermal hysteresis has been recorded in a number of polar terrestrial
166 invertebrates, including Antarctic and Arctic mites (*A. antarcticus* [Block *et al.* 2009] and *Phauloppia*
167 sp. [Sjursen and Sømme 2000]), Antarctic Collembola (e.g. *C. antarcticus* [Block *et al.* 2009] and
168 *Gressittacantha terranova* [Hawes *et al.* 2011]), Alaskan beetles (e.g. *Cucujus clavipes* [Sformo *et al.*
169 2010]) and Alaskan lacewings (e.g. *Hemerobius simulans* [Duman *et al.* 2004]). AFPs provide further
170 protection by stabilising the supercooled state and preventing inoculative freezing (Bale 1993), and
171 preserving membranes during phase transitions (Duman *et al.* 2004). In a similar manner to freeze-
172 tolerant species, freeze-avoiding invertebrates also utilise polyols, sugars and amino acids for

cryoprotection and the relative enhancement of metabolism at lower temperatures (Block *et al.* 2009; Clark and Worland 2008; Muise and Storey 2001). Polyols and sugars also help to lower the SCP in a non-colligative manner like AFPs (Lee *et al.* 1996).

2.3. Commonalities between freeze-tolerance and freeze-avoidance

2.3.1. Responses to chilling injury

Freeze-tolerance and freeze-avoidance are mechanistically distinct from each other. However, there is also commonality between the two strategies, as organisms deploying them are both susceptible to, and therefore must also guard against, chilling injury. Chilling is defined as cooling sufficient to induce damaging effects or even death in the absence of freezing (Hayward *et al*. 2014). Extreme chilling injury can result from rapid cooling (cold shock or acute stress), as well as long-term exposure to low temperatures (chronic stress) and/or experience of temperature extremes (Czajka 1990). In truth, chilling and cold stress are relative terms, and the temperatures at which they occur will depend on multiple factors, ranging from the species' evolutionary history and geographic origin, to an individual's physiological status and recent thermal history. Chilling-induced damage includes the loss of integrity, fluidity, and thus function, of the membrane (Izumi *et al*. 2009), the deterioration of intracellular organelles (Strange and Dark 1962), the disruption of enzymes and electrochemical ion potentials (Denlinger and Lee 2010), and the destruction of whole cells through apoptosis (Yi *et al.* 2007).

The plasma membranes which surround cells of all life forms allow for the selective transfer of solutes across the cell, intra- and inter-cellular communication, the application of energy harnessed through transmembrane ion gradients, and function as a barrier to pathogens and toxins (Hazel 1995). It is therefore necessary for plants, microbes and animals, including invertebrates, to maintain membrane fluidity and function as temperature falls. Homeoviscous adaptation is a process which permits the maintenance of membrane fluidity, in particular through alterations in the composition of membrane phospholipid fatty acid chains (Hazel 1995). Desaturase enzymes are known to play a fundamental role in membrane responses to low temperature, by raising the number of unsaturated fatty acids (UFAs) relative to saturated fatty acids (SFAs) (Murray *et al*. 2007; Hayward *et al.* 2007). These UFAs introduce more double bonds (or kinks) into the phospholipid matrix and so reduce phospholipid aggregation. The fluidity of the membrane and the transition phase (Tm = fluid to gel) are also influenced by the position of double bonds and the length of fatty acid chains (Baenzinger *et al.* 1992), and some invertebrates, including *M. arctica*, respond by augmenting these attributes (Bahrndorff *et al.* 2007).

HSPs also play an important role in response to chilling injury, as they are involved in refolding and stabilising denatured proteins, recovering microfilament dynamics and regulating apoptosis at low temperatures (Benoit and Lopez-Martinez 2012; Clark and Worland 2008; Tammariello *et al.* 1999; Yi *et al.* 2007). HSPs have been shown to be constitutively expressed in larva of the Antarctic midge, *B. antarctica*, which experience chronic cold, while adults, found in more variable environments, only expressed HSPs as a direct response to stress (Rinehart *et al.* 2006). The latter scenario represents the 'classic' HSP response, as seen in non-polar species like the potato beetle, *Leptinotarsa decemlineata* (Yocum 2001) and the onion fly, *Delia antiqua* (Chen *et al.* 2006), amongst others. Direct evidence of their contribution to stress tolerance has also been shown in the firebug, *Pyrrhocoris apterus* (Kostal and Tollarova-Borowanska 2009), and the flesh fly, *S. crassipalpis* (Rinehart *et al.* 2007). In both species, injection with *hsp70* double-stranded RNA (RNAi) was used to suppress *hsp70* mRNA and

protein levels, which resulted in reduced survival (Rinehart *et al.* 2007), or restricted ability to repair chilling injury and mate successfully (Kostal and Tollarova-Borowanska 2009).

Further mechanisms suggested to play roles in chilling injury include mitochondrial degradation, which reduces metabolism and energy use (Levin *et al.* 2003), the accumulation of polyols and sugars, which act as cryoprotectants (Montiel *et al.* 1998), and the build-up of amino acids, which may serve as a reserve for HSPs or stabilise supercooling (Kostal *et al.* 2011). A modification of the schematic representation of freeze-avoidance and freeze-tolerance strategies of Bale (2002), which now includes the molecular and physiological responses to chilling (common to both strategies), is presented in Fig. 1.

2.3.2. Vitrification

Under crystalline conditions, cells eventually degrade and lose their viability when exposed to chronic chilling or cold shock (Katkov 2006). Freezing can also irreversibly damage cells. Vitrification (or the attainment of a glass-like state) in contrast does not lead to such injuries. Vitrified fluids behave more like a solid and yet also show little change in their molecular state. Under these conditions, an organism's fluids are stable and immune from molecular degradation. It is speculated that vitrification could further aid freeze-avoidance and freeze-tolerance strategies of polar terrestrial invertebrates. Clarke *et al.* (2013) concluded that vitrification occurs in a number of unicellular organisms, including bacteria, and photosynthetic and heterotrophic eukaryotes, when cooled slowly in the presence of extracellular ice. Cellular dehydration resulting from the movement of water out of the cell to join newly formed ice crystals and the subsequent increase in fluid viscosity inside the cell, under slow rates of cooling, allows these unicellular organisms to vitrify prior to intracellular freezing.

Clarke *et al.* (2013) hypothesised that vitrification would also occur in the cells of freeze-tolerant multicellular organisms, where ice formation in the extracellular space, rather than in the environment, would encourage intracellular dehydration and the consequential formation of a glass state. As an example, Clarke *et al.* (2013) postulated that the Alaskan tenebrionid beetle, *Upis ceramboides*, would vitrify. *Upis ceramboides* freezes at high sub-zero temperatures and is able to survive in this frozen state to -60°C (Miller 1978). It is likely that the dehydration induced by extracellular ice formation would eventually also lead to glass formation, and would only be limited by the rate at which the beetle is cooled (Miller 1978).

In the presence of ice in the environment, several polar freeze-avoiding organisms are known to desiccate in a process called cryoprotective dehydration, e.g. the beetle *Cucujus clavipes* (Bennet *et al.* 2005) and *M. arctica* (Worland *et al.* 1998). These organisms also accumulate a number of polyhydric alcohols and sugars, such as trehalose, which increase the viscosity of cellular fluid. It has thus previously been speculated that they may also be capable of vitrification. A recent study (Sformo *et al.* 2010) indicates that this is the case, having shown the Arctic red flat bark beetle, *C. clavipes puniceus*, to vitrify at a very low temperature (-58°C). As a consequence, 43% of the beetles were able to survive between -70 and -73°C, and a further 7% were able to survive -100°C (Sformo *et al.* 2010). Although the ecological relevance of vitrification in this beetle is questionable, with temperatures in Alaska rarely falling to -58°C, confirmation of the presence of this trait is significant.

2.3.3. Acclimation and cooling rates

The variation in temperature between summer and winter in the Antarctic and Arctic is great, and temperatures annually can vary by tens of degrees in buffered microhabitats, and by as much as 100^{o}C

on exposed soil and rock surfaces (Convey 1996). There are some invertebrates, such as the nunatak inhabiting springtail, *Cryptopygus sverdrupi* (Sømme 1986), which manage this scenario by remaining in a cold hardy state all year round. Larvae of *B. antarctica* also constitutively express Hs (Rinehart *et al*. 2006). However, the vast majority of polar invertebrates are not in a constant state of readiness for the winter months and instead acclimatise (Deere *et al.* 2006), preparing themselves physiologically and improving their low temperature tolerance in the lead-up to winter. Examples of acclimation have been shown in the chironomid *E. murphyi* (Worland 2010), the beetle, *Hydromedion sparsutum* (Bale *et al.* 2000), the aphid, *Myzus polaris* (Hazell *et al.* 2010), the mite *H. belgicae* (Hawes *et al.* 2007), the collembolan, *C. antarcticus* (Worland *et al.* 2007), the nematode, *Plectus murrayi* (Adhikari *et al.* 2010), and a number of other polar invertebrates (and also temperate invertebrates, which similarly experience a distinct summer to winter transition). The change in temperature from summer to winter is gradual, allowing evolution to optimise the rate at which invertebrates acclimatise to this slow rate of cooling. It is well known that faster cooling rates reduce the survival of freeze-tolerant species, raise the SCP of freeze-avoiding species, and reduce the capacity of these animals to respond to chilling injury (Sinclair *et al.* 2003).

Acclimation to low temperatures can also occur on a shorter timescale of minutes to hours via rapid cold hardening (RCH) (Lee *et al.* 2006a; Yi *et al.* 2007). RCH was first described in the flesh fly, *S. crassipalpis* (Lee *et al.* 1987), and has since been observed in a wide range of other organisms, including polar species such as *B. antarctica* (Lee *et al.* 2006a), *E. murphyi* (Everatt *et al.* 2012), *H. belgicae* (Hawes *et al.* 2007; Worland and Convey 2001), *C. antarcticus* and the mite, *Alaskozetes antarcticus* (Worland and Convey 2001). The response generally provides only moderate survivorship benefits, however, with survival typically being extended for, at most, 10 hours at the discriminating temperature (DT = temperature at which there is between 10 and 20% survival upon exposure for 2 hours), or lowering the DT by just 2-3^{o}C (Bale 2002). However, there are exceptions. Following RCH, the LLT of *E. murphyi* larvae decreased by up to 6.5^{o}C, and survival of larvae of the same species was maintained above 80% for at least 22 h at the DT (Everatt *et al.* 2012).

RCH also impacts on sub-lethal characteristics, including at temperatures above 0°C. In the fruit fly, *Drosophila melanogaster*, courting and reproduction were 35 and 55% greater at 16^{o}C, respectively, following RCH (Shreve *et al.* 2004). Further sub-lethal improvements have included the maintenance of the proboscis extension reflex and grooming behaviour in flesh flies (Kelty *et al.* 1996), the preservation of learning and spatial conditioning (Kim *et al.* 2005), and the sustenance of flight (Larsen and Lee 1994). Similar improvements are likely to be found in polar invertebrates though, as yet, they have not been explored.

The survival and behavioural improvements of RCH are likely to be highly advantageous and may allow invertebrates to adjust quickly to, and track, environmental temperatures on both a temporal (daily) and spatial (microhabitat) scale (Kelty and Lee 1999; Powell and Bale 2004, 2005, 2006; Shreve *et al.* 2004; Worland and Convey 2001). Analogous to acclimation over weeks and months, a gradual rate of cooling that is more in line with nature tends to elicit greater protection (Chidawanyika and Terblanche 2011; Kelty and Lee 1999, 2001; McDonald *et al.* 1997; Wang and Kang 2003). As suggested by Wang and Kang (2003) and others, this enhanced protection is presumably because of the greater time these individuals spend at protection-inducing temperatures.

Although the ecological role of RCH is well established, relatively little is known about the physiological mechanisms underlying the response. Recent studies suggest that RCH is driven by a calcium signalling cascade involving calmodulin, which allows cells to sense changes in temperature and trigger downstream physiological responses (Teets *et al.* 2008). Protection against cold-induced

apoptosis is likely to be one such physiological response. RCH is able to impair apoptosis by down-regulating promoters of the response and up-regulating apoptosis inhibitors. In *D. melanogaster* and *S. crassipalpis*, apoptosis was reduced by >34% following RCH (Yi *et al.* 2007; Yi and Lee 2011). RCH also involves a homeoviscous adaptation response. With the use of solid state NMR spectroscopy, Lee *et al.* (2006b) were able to demonstrate enhanced membrane unsaturation and a subsequent rise in membrane fluidity in *B. antarctica* following RCH. Metabolic adjustments, including the accumulation of polyols and sugars during RCH and the minimisation of metabolic perturbations during cold shock recovery, may likewise play a key role (Michaud and Denlinger 2007; Overgaard *et al.* 2007; Teets *et al.* 2012). However, the universality of homeoviscous adaptation and metabolic adjustment is in question, as some invertebrates show an RCH response in the absence of either the elevation of polyols and sugars or alterations in membrane composition (MacMillan *et al.* 2009). Because apoptosis inhibition and homeoviscous adaptation, as well as metabolic adjustments to a large degree, concern responses to chilling injury, this suggests that chilling injury, rather than freezing damage, is the primary target of RCH in the chill-susceptible and freeze-tolerant invertebrates studied. The same cannot be said for some freeze-avoiding invertebrates, however, such as *C. antarcticus*, in which the SCP is lowered during RCH (Worland and Convey 2001). Worland and Convey (2001) also confirmed that the body water content and solute concentration of freeze-avoiding invertebrates were unaffected by RCH, and hypothesised that RCH in these organisms could be understood by the inhibition of INAs, though this remains unconfirmed.

3. Responses to low water availability

Water is a requirement for all life on Earth (Hodkinson *et al.* 1999). Without it, living organisms are exposed to desiccation and its associated injuries, which include protein denaturation and unwanted macromolecular interactions (Sano *et al.* 1999; Tang and Pikal 2005), crystalline to gel membrane phase transitions (Hazel 1995), oxidative damage (Lopez-Martinez *et al.* 2008), mechanical stress and the rapid influx of water following rehydration (Bayley and Holmstrup 1999). The possibility of such injuries is particularly high in the Antarctic and Arctic, where water is unavailable for extended periods of the year (Block *et al.* 2009). Invertebrates protect against this threat physiologically through the adoption of one of two strategies, desiccation resistance or desiccation tolerance (Fig. 2, Danks 2000).

3.1. Desiccation resistance

Desiccation resistance is defined as the capacity to prevent water loss from the body. The extent to which this occurs varies greatly amongst polar invertebrates (and invertebrates in general), leading to the recognition of three groups - hygric, which have little or no control over their water loss, transitional and mesic, which are increasingly able to regulate the loss of their body water (Eisenbeis 1983). The mesic status of some invertebrates is partly due to their lowered cuticular permeability. Reduced permeability is largely achieved through the modulation of the wax layer, which coats the cuticle and consists of bipolar molecules with hydrophobic and hydrophilic ends (Speight *et al.* 2008). In the majority of invertebrates, the hydrophobic ends face outward and limit the rate of water loss. However, mesic species go a little further and tend to either accumulate or increase the length of hydrocarbons or hydrophobic molecules, resulting in tighter packing and a greater reduction of water loss (Benoit *et al.* 2007a). For instance, the mesic mite, *A. antarcticus*, experienced a lower rate of water loss than the mites, *Hydrogamasellus antarcticus* and *Rhagidia gerlachei*, which had two to three times less hydrocarbons. *A. antarcticus* was also shown to have a high critical transition temperature of 25^{o}C, below which hydrocarbons remained stable and cells remained relatively watertight (Benoit *et al.* 2007a).

The accumulation of polyols and sugars, and subsequent absorption of water, has also proven a beneficial strategy in a number of Antarctic species, such as *C. antarcticus* (Elnitsky *et al.* 2008b) and *B. antarctica* (Benoit *et al.* 2009a), which are able to depress the rate of water loss through the accumulation of osmolytes. Perhaps the best example of this is given by the non-polar collembolan, *Folsomia candida*. Having lost almost half of its osmotically active water under 98.2% RH, this collembolan was able to recover nearly all of the loss within 5-7 d, via the accumulation and synthesis of myo-inositol, glucose and trehalose (Bayley and Holmstrup 1999; Timmermans *et al.* 2009). There are also some species, including astigmatid mites, that are able to maintain an equilibrium with the environment at between 70 and 98% RH from the outset (Benoit *et al.* 2007a, 2009a). Further means of resisting desiccation are freezing (Convey 1992), membrane alterations and metabolic suppression (Michaud *et al.* 2008) as well as specialised respiration (Convey *et al.* 2003; Danks 2000; Slama 1988).

Further, recent studies on species of *Drosophila* have shown a correlation between body melanisation and desiccation resistance, which corresponds with the aridity of the flies' local climate. Parkash *et al.* (2008a, b) demonstrated greater desiccation resistance in darker morphs of *Drosophila melanogaster* and *D. immigrans* that were predominantly found in drier, high altitude habitats, as compared with their lighter counterparts found at lower altitudes. Likewise, lower desiccation resistance of *D. melanogaster* and *D. ananassae* during the rainy season was correlated with lower melanisation (Parkash *et al.* 2009, 2012). Greater desiccation resistance in strains of *D. melanogaster* selected for higher levels of melanisation, and the reverse in those selected for lower levels, has now provided direct evidence of the phenomenon (Ramniwas *et al.* 2013).

3.2. Desiccation tolerance

For polar invertebrates that are less desiccation resistant, an ability to tolerate water loss is crucial for survival (Danks 2000). Some invertebrates are particularly tolerant - *Dendrobaena octaedra* cocoons (Holmstrup and Zachariassen 1996) and larvae of *B. antarctica* (Hayward *et al.* 2007) are able to endure >75% loss of their water content, and some nematodes and tardigrades are able to survive the loss of virtually all of their osmotically active water and most of their osmotically inactive water in a process called anhydrobiosis during which metabolism ceases (Crowe and Madin 1975; Hengherr *et al.* 2010; Watanabe *et al.* 2002; Wharton 1993, 2003b, 2011; Wharton and Worland 2001). The mechanisms which these organisms utilise to confer tolerance are many and include the accumulation of polyols and sugars, the activation of HSPs and Late Embryogenesis Abundant (LEA) proteins, membrane remodelling and oxidative damage repair (Gusev *et al.* 2010; Watanabe 2006).

3.2.1. Polyols and sugars

Polyols and sugars are produced in response to desiccation in a number of polar invertebrate groups, including nematodes (e.g. *Plectus murrayi* [Adhikari *et al.* 2010]), midges (e.g. *B. antarctica* [Benoit *et al.* 2009a]), beetles (e.g. *H. sparsutum* [Bale *et al.* 2000]) and Collembola (e.g. *C. antarcticus* [Elnitsky *et al.* 2008b]). Of these, *B. antarctica* has been especially well studied. As a result of water loss and *de novo* synthesis, larvae of this species raise the level of glycerol and trehalose two to three fold (Benoit *et al.* 2007b). Two hypotheses have been put forward for the function of polyols and sugars during desiccation. The first is that polyols and sugars, particularly trehalose, are used for the replacement of water. Subsequently, cellular damage and deleterious protein interactions, which would otherwise occur in the absence of water, are reduced (Benoit *et al.* 2009a). The second hypothesises is that the low molecular weight compounds aid the production of amorphous sugar glasses (e.g. through the formation of hydrogen bonds with membrane phospholipids [Sakurai *et al.*

2008]). These glasses stabilise proteins and membranes by minimising physical and chemical reactions involving molecular diffusion, such as solute crystal nucleation (Bahrndorff *et al.* 2009; Danks 2000; Hengherr *et al.* 2009). It should also be noted that the mechanisms responsible for the survival of desiccation tolerance are not necessarily universal. There are some anhydrobiotic invertebrates, such as the tardigrade, *Milnesium tardigradum* (Hengherr *et al.* 2008) and rotifers (Caprioli *et al.* 2004; Lapinski and Tunnacliffe 2003), which do not seem to accumulate trehalose or any other carbohydrate molecules. It has been suggested, because of the presence of anhydrobiosis in many taxonomic groups, that the ability has evolved several times and, during each evolutionary event, different mechanisms have been exploited (Alpert 2006).

3.2.2. HSPs and LEA proteins

Protein denaturation is a common injury following desiccation. HSPs are induced in direct response to protein damage, and are well recognised as being involved in the reformation or degradation of affected proteins (Benoit and Lopez-Martinez 2012; Feder and Hofmann 1999). Thus, it is unsurprising that HSPs are up-regulated in response to desiccation in several invertebrates, including tardigrades (Hengherr *et al.* 2008), Collembola (Timmermans *et al.* 2009) and Antarctic midges (Lopez-Martinez *et al.* 2009). The group of proteins known as LEA proteins has also been shown to play a role in desiccation tolerance in a number of invertebrates, including polar species (Bahrndorff *et al.* 2009; Browne *et al.* 2002, 2004; Gal *et al.* 2004; Goyal *et al.* 2005; Watanabe *et al.* 2003). LEA proteins possess many of the same attributes as HSPs, being able to prevent protein aggregation and preserve enzymatic activity. These proteins may also be able to suppress unwanted macromolecular interactions and maintain membrane fluidity *in vitro* (Bahrndorff *et al.* 2009), reduce water loss, prevent ice crystal formation (Bokor *et al.* 2005) and stabilise sugar glasses (Wolkers *et al.* 2001). There is even evidence that LEA proteins are fragmented into smaller, but still functional, components in response to increased desiccation and are thereby better able to counteract damage (Kikiwada *et al.* 2006).

3.2.3. Membrane remodelling

As with low temperature, the loss of water from cells and membranes leads to the transition of the plasma membrane from a crystalline to a gel phase (Hazel 1995). Some invertebrates ameliorate this stress via homeoviscous adaptation. In *B. antarctica*, enzymes such as Δ9 FAD and fatty acyl CoA Δ9 desaturases are used to increase unsaturation, and thus also fluidity, of the membrane (Lopez-Martinez *et al.* 2009). However, membrane remodelling in this midge may also involve the replacement of unsaturated membrane fatty acids with saturated forms. Although seemingly counterintuitive, this substitution minimises the impact of singlet oxygen, a product of desiccation, which reacts directly with polyunsaturated fatty acid side chains and subsequently causes lipid peroxidation (Lopez-Martinez *et al.* 2009).

3.2.4. Oxidative damage repair

Desiccation of invertebrates results in the production of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide radicals. ROS cause numerous injuries, including the disruption of membrane fluidity, apoptosis of mitochondria, denaturation of proteins and fragmentation of DNA (Lopez-Martinez *et al.* 2008). Antioxidants, primarily superoxide dismutase (SOD), catalase and glutathione peroxidase, are accumulated in organisms in response to these injuries and inhibit ROS. Such accumulation has been observed in a number of organisms, including plants (Jenks and Wood 2007), nematodes (Reardon *et al.* 2010) and the Antarctic midge, *B. antarctica* (Lopez-Martinez *et al.*

2008). However, antioxidants are unable to completely arrest the effects of oxidation in some species. There is therefore a need for other defences, such as apoptosis of damaged cells or a DNA repair system. The latter is used in the midge, *Polypedilum vanderplanki*, and is achieved through the up-regulation of Rad23 and Rad51, which are genes associated with the repair of DNA breaks (Gusev *et al.* 2010).

3.2.5. Additional mechanisms

There are several other processes known to be involved in desiccation tolerance which may be utilised by polar invertebrates. These include cytoskeletal reorganization, such as the synthesis of actin, tropomyosin and myosin for the maintenance of the cytoskeleton (Lopez-Martinez *et al.* 2009; Li *et al.* 2009), the accumulation of aquaporins for the efficient transport of water and solutes from and into the body (Li *et al.* 2009; Philip *et al.* 2008, 2010), the removal and redistribution of osmolytes during rehydration (Bayley and Holmstrup 1999; Hayward *et al.* 2007), the regulation of autophagy (Teets *et al.* 2012), the down-regulation of metabolism and ATP production (Teets *et al.* 2012), and the possession of a high initial water content (Hayward *et al.* 2007).

4. Links between low water availability and low temperature tolerance

The responses of invertebrates to low temperature and low water availability are not exclusive, and there is considerable linkage between the two. This linkage is especially apparent in environments which frequently experience both stresses sequentially or in tandem. The fellfields of the Antarctic and tundra of the Arctic are prime examples of such environments (Block *et al.* 2009; Strathdee and Bale 1998).

4.1. Cross-tolerance following stress acclimation

There is increasing awareness that a sub-lethal exposure of an invertebrate to low relative humidity (RH) not only improves tolerance to low RH, but also to low temperature. This phenomenon can now be understood through the interrelationship that exists between low temperature and low water availability. Both stressors result in similar injuries and physiological challenges, including reduction of the fluidity, and thus stability and function, of plasma membranes (Bayley *et al.* 2001), impairment of protein folding (Ring and Danks 1994) and, in the case of freezing, increase of pH and osmolality of cellular fluid (Ring and Danks 1994). It therefore follows that the physiological mechanisms induced by an invertebrate in response to these stresses are also similar, or at least complementary. Several studies have suggested that the mechanisms used in response to low temperature evolved from those used in response to low water availability either as aquatic organisms colonised the land, or as they moved from generally less stressful tropical and temperate latitudes towards the poles (Block 1996; Ring and Danks 1994, 1998).

4.1.1. Polar examples

Perhaps the best-described example of cross-tolerance in a polar terrestrial invertebrate is that in the flightless Antarctic midge, *B. antarctica.* Following desiccation at 0-98.2% RH, larvae of *B. antarctica* show significantly higher survival at -10 and -15^{o}C, as compared with fully hydrated larvae (Benoit *et al.* 2009a; Hayward *et al.* 2007). There is now also evidence that the closely related Antarctic midge, *E. murphyi*, and the Antarctic nematode, *Plectus murrayi*, are capable of cross-tolerance, with significantly improved survival of *E. murphyi* larvae at -18^{o}C, and enhanced survival of the nematode at -10^{o}C, following desiccation (Adhikari *et al.* 2010; Everatt *et al.* in press). Similar examples of cross-tolerance are found at lower latitudes in the goldenrod gall fly, *Eurosta solidaginis*,

478 which exhibits reduced water loss after low temperature acclimation, and in the collembolan, *F.*
479 *candida*, which shows enhanced low temperature tolerance after acclimation at 98.2% RH (Holmstrup
480 *et al.* 2002; Levis *et al.* 2012; Williams and Lee 2008).

481 *4.1.2. Mechanisms of cross-tolerance*

482 In *B. antarctica*, several physiological mechanisms that are common in response to both low
483 temperature and desiccation have been proposed to underlie cross-tolerance. Following a preliminary
484 analysis of desiccation-responsive solutes using Fourier Transform Infrared (FT-IR) spectroscopy and
485 discrimination function analysis, Hayward *et al.* (2007) found the polysaccharide region of the spectra
486 to change considerably. They went on to suggest that polyols and sugars, which serve as cryo- and
487 osmo-protectants, play an important role in conferring cross-tolerance. Benoit *et al.* (2007b, 2009a)
488 further demonstrated the level of glycerol and trehalose to increase in larvae of *B. antarctica*
489 following exposure to 75 and 98% RH, and later showed, by injecting exogenous trehalose into
490 larvae, that an enhanced concentration of trehalose leads to greater low temperature tolerance. Shared
491 metabolites produced in response to desiccation and low temperature were also shown in larvae of *B.*
492 *antarctica* by Michaud *et al.* (2008). These studies show that the accumulation of polyols and sugars
493 at the organismal level are important in desiccation and cross-tolerance. However, tolerance in *B.*
494 *antarctica* may not be as straightforward as a simple accumulation. Williams and Lee (2011) have
495 demonstrated that glycerol and sorbitol are not only accumulated in *E. solidaginis*, but are also moved
496 from extra- to intra-cellular fluids during exposure to low temperature or desiccation. This movement
497 may be especially important in freeze-tolerant invertebrates, such as *B. antarctica*, in which cells lose
498 water to the extracellular space and become susceptible to injuries associated with desiccation.
499 Polyols and sugars may subsequently act to replace water or aid in the formation of amorphous glass
500 inside the cell (Calderon *et al.* 2009). The transfer of these solutes across the cellular membrane is
501 likely orchestrated by water- and small uncharged solute specific-channels known as aquaporins or
502 aquaglyceroporins. These were first identified in human kidney and red blood cells, but have since
503 been found in yeast, plants, arthropods, birds, anurans and non-human mammals (Beuron *et al.* 1995;
504 Muller *et al.* 2006; Nielsen *et al.* 1993; Preston *et al.* 1992). Using the aquaporin inhibitor, mercuric
505 chloride ($HgCL_2$), Izumi *et al.* (2006) and Philip *et al.* (2008) demonstrated reduced freezing survival
506 and therefore a direct role of aquaporins in the rice stem borer, *Chilo suppressalis*, and *E. solidaginis*,
507 respectively. Philip and Lee (2010) further confirmed the role of aquaporins by showing that AQP3-
508 like proteins and AQP4-like proteins increase from summer to winter in *E. solidaginis* following low
509 temperature initiation.

510 Further mechanisms which have been suggested as important in the cross-tolerance of *B. antarctica*
511 include heat shock proteins (HSPs) and antioxidants. Although HSPs (smHsp, Hsp70 and Hsp90) are
512 constitutively expressed in larvae of *B. antarctica*, both slow and rapid desiccation are able to further
513 up-regulate them (Lopez-Martinez *et al.* 2009; Teets *et al.* 2012). HSPs are used during stress to
514 reform or degrade damaged proteins and other macromolecular structures. It is plausible that up-
515 regulation of these proteins following desiccation could repair proteins at low temperature and
516 improve low temperature tolerance (or *vice versa*). A caveat to this hypothesis is that, in *S.*
517 *crassipalpis*, the up-regulation of HSP transcripts after desiccation did not provide any further
518 tolerance to low temperature (Tammariello *et al.* 1999). However, it should be recognised that
519 transcript up-regulation is not direct evidence of protein synthesis. Thus, it remains unclear if the
520 utilisation of HSPs is a cross-tolerance mechanism universally utilised by invertebrates. LEA proteins
521 are also up-regulated under desiccation in a number of invertebrates, such as nematodes, rotifers,
522 chironomids and Collembola (Bahrndorff *et al.* 2009; Browne *et al.* 2002; Kikiwada *et al.* 2006;

Tunnacliffe *et al.* 2005). It has therefore been suggested that they may likewise play a role in cross-tolerance, as has already been established for a number of plant species (Kosova *et al.* 2007).

The loss of water under freezing and desiccation leads to the production of ROS, and Lopez-Martinez *et al.* (2008, 2009) have shown that antioxidants and detoxification enzymes, including superoxide dismutase, catalase, metallothionein and cytochrome P450 monoxygenase, are up-regulated in response to desiccation in larvae of *B. antarctica*. These same antioxidants and detoxification enzymes would likely provide similar protection against oxidative damage in larvae that are frozen.

Similar physiological symptoms are also seen with respect to the membrane. Under desiccation and low temperature, phospholipid fatty acid chains of the membrane pack together, resulting in the loss of membrane fluidity and function (Hazel 1995). Most invertebrates arrest these changes by raising the level of unsaturation of phospholipid fatty acids and introducing kinks into the membrane (Hazel 1995). In *B. antarctica*, evidence of unsaturation is shown in relation to both desiccation (Lopez-Martinez *et al.* 2009) and low temperature (Lee *et al.* 2006b). It is therefore likely that the unsaturation induced in response to one stress may provide protection in response to the other stress. Further support for this hypothesis is provided by the collembolan, *F. candida*. Unsaturation of phospholipid fatty acids takes place under desiccation (98.2% RH) in this species but, importantly, the time spent at 98.2% RH which leads to a significant change in unsaturation (196 h) is the same as that required at 98.2% RH to give a significant change in low temperature tolerance (Bayley *et al.* 2001; Holmstrup *et al.* 2002).

The sub- Antarctic flightless midge *E. murphyi* is freeze-tolerant and is hypothesised to use similar cross-tolerance mechanisms to *B. antarctica*. This is plausible, not only based on their close phylogenetic relationship (Allegrucci *et al.* 2006, 2012), but also because the physiological comparisons to date have been analogous. In particular, their low temperature tolerance and rapid cold hardening responses are very similar (Everatt *et al.* 2012; Lee *et al.* 2006).

4.2. Cross-tolerance following anhydrobiosis

Examples of improved low temperature tolerance have so far been restricted to invertebrates experiencing only partial desiccation. However, there are a number of invertebrates which are capable of surviving the loss of virtually all of their body water (Crowe and Madin 1975). These invertebrates are said to be cryptobiotic, or more specifically, anhydrobiotic (Sømme 1996), and include polar nematodes (Treonis *et al.* 2000; Wharton and Barclay 1993; Wharton 2003b, 2011a), and non-polar crustaceans (Crowe and Clegg 1973), rotifers (Tunnacliffe and Lapinski 2003), tardigrades (Hengherr *et al.* 2008), and midge larvae (Gusev *et al.* 2010; Sakurai *et al.* 2008). When in a state of anhydrobiosis, such invertebrates show a remarkable ability to survive low temperature. The tardigrade, *Adorybiotus coronifer*, for example, is able to survive cooling to -196^{o}C (Ramløv and Westh 1992), while others are able to survive in liquid helium (-271°C) (Shuker 2001). Invertebrates like tardigrades can also survive extreme conditions even in their hydrated state. The Antarctic tardigrades, *Echiniscus jenningsi*, *Macrobiotus furciger* and *Diphascon chilenense*, show survival after 590 days at -22^{o}C, good survival at -80^{o}C and even some survival at -180^{o}C. Surprisingly when anhydrobiotic, tolerance of low temperatures is actually attenuated in these species (Sømme and Meier 1995).

The mechanisms which anhydrobiotic invertebrates use to survive the loss of all their osmotically active water and confer tolerance at low temperature may be similar to those previously described, though it is postulated that, because the level of desiccation is greater, the strength of these

mechanisms may be greater also. The accumulation of polyols and sugars, particularly trehalose, conform to this hypothesis in a number of anhydrobiotic invertebrates, including nematodes (Crowe and Madin 1975; Madin and Crowe 1975), midge larvae (Watanabe *et al.* 2002) and tardigrades (Ramløv and Westh 1992). In the tardigrade, *A. coronifer*, and members of the Macrobiotidae family, the accumulation during entry into anhydrobiosis is particularly pronounced, increasing the level of trehalose by up to 20-fold (Hengherr *et al.* 2008; Ramløv and Westh 1992). Increases in trehalose found in *B. antarctica* are, in contrast, less obvious (Benoit *et al.* 2007b). The role of polyols and sugars, as with partially-desiccated invertebrates, has been attributed to water replacement and/or vitrification (Danks 2000). The latter is now seen as being essential in some anhydrobiotic invertebrates. Using FTIR analysis and differential scanning calorimetry (DSC), Sakurai *et al.* (2008) show larvae of the midge *P. vanderplaanki* to form a glass-like state during anhydrobiosis. Significantly, larvae were unable to successfully rehydrate when artificially taken out of this state.

4.3. Cross-tolerance following selection

To examine whether cross-tolerance to one stress could be enhanced by selecting for improved basal tolerance to another stress (i.e. without prior acclimation), Bubliy and Loeschcke (2005) artificially selected for either cold or desiccation tolerance in the fruit fly, *D. melanogaster*, and subsequently exposed selected individuals to the opposing stress. They found that individuals selected for desiccation resistance had greater low temperature tolerance at 0.5^{o}C. Sinclair *et al.* (2007) also found that selection for desiccation resistance in *D. melanogaster* resulted in a decreased chill coma recovery time, approximately 2 min less (15% reduction) than control flies. However, Sinclair *et al.* (2007) did not find a discernible improvement in the low temperature survival of *D. melanogaster* following desiccation resistance selection. Likewise, selection for low temperature tolerance did not result in an improvement in the flies' desiccation resistance/tolerance (MacMillan *et al.* 2009). The lack of cross-tolerance was suggested by MacMillan *et al.* (2009) to indicate that changes in basal tolerance are not responsible for cross-tolerance. Yet, because there are some studies, including those mentioned (e.g. Bubliy and Loeschcke 2005), which do show cross-tolerance through selection of basal tolerance, it was also suggested that other selective pressures as part of the experimental design may act to break up linkage gene groups associated with multiple stress tolerance (MacMillan *et al.* 2009). As outlined in this review, multiple physiological processes underpin both cold and desiccation tolerance, and artificial selection experiments are unlikely to target all the underlying genetic mechanisms.

4.4. Cryoprotective dehydration

Cryoprotective dehydration was first discovered by Holmstrup (1992) in cocoons of the earthworm, *Dendrobaena octaedra*, and has since been described in nematodes (e.g. *Panagrolaimus davidi* [Smith *et al.* 2008]), enchytraeid worms (e.g. *Fridericia ratzeli* [Pedersen and Holmstrup 2003]), Collembola (Elnitsky *et al.* 2008b; Sorensen and Holmstrup 2011; Worland *et al.* 1998) and even mammalian cells (Pegg 2001). This process occurs in an environment in equilibrium with the vapour pressure of ice. Under these conditions, invertebrates continue to lose water along a diffusion gradient between their supercooled body fluids and the surrounding ice until the vapour pressure of their body fluids is equal to that of the environment (Wharton *et al.* 2003a). The subsequent concentration and *de novo* synthesis of solutes (Elnitsky *et al.* 2008a) causes the SCPs of invertebrates to be reduced and their MPs to become equilibrated with the ambient temperature (Elnitsky *et al.* 2008a; Holmstrup *et al.* 2002; Pedersen and Holmstrup 2003). In this state, the risk of freezing is eliminated (Elnitsky *et al.* 2008).

Cryoprotective dehydration is perhaps best exemplified in the Arctic collembolan, *M. arctica*. The response was first described in this collembolan by Worland *et al.* (1998) and Holmstrup and Sømme (1998), who showed the SCP of *M. arctica* to fall as low as -30°C when the temperature was reduced to -12.4°C. The melting point (MP) was also shown to decrease with temperature (Holmstrup and Sømme 1998), and was later shown by Holmstrup *et al.* (2002) to decline in parallel with the environmental temperature, before equilibrating with this temperature after a 1-6 day lag period. Cryoprotective dehydration is not restricted to freeze-avoiding invertebrates such as *M. arctica*, but also extends to freeze-tolerant species. For instance, larvae of *B. antarctica* have been demonstrated to lose water in the presence of ice when cooled to -3°C, and have subsequently shown to have a three-fold depression of their MP (Elnitsky *et al.* 2008a).

The solutes accumulated during cryoprotective dehydration are similar in *B. antarctica* and *M. arctica* and include glucose and trehalose (Elnitsky *et al.* 2008a; Holmstrup *et al.* 2002). Glucose is likewise accumulated during cryoprotective dehydration in the earthworm, *F. ratzeli* (Pedersen and Holmstrup 2003). Other polyols, sugars and amino acids may also be involved in the process (Elnitsky *et al.* 2008a). The accumulation of these solutes has already been shown to lower the SCP and MP, and they may also lead to a vitrified state, as has been shown in at least one invertebrate, the red flat bark beetle, *C. c. puniceus* (Sformo *et al.* 2010).

For cryoprotective dehydration to be a viable strategy, invertebrates must possess low desiccation resistance (cuticular permeability) and high desiccation tolerance (Bahrndorff *et al.* 2007). The former is required for the transport of water from the supercooled body fluids to the external environment at a rate equivalent to that of the lowering of ice vapour pressure with temperature (Holmstrup *et al.* 2002), while desiccation tolerance is imperative if the organism is to survive considerable water loss.

5. Links between low water availability and high temperature tolerance, and tolerance to other stresses

Cross-tolerance between desiccation and high temperature has been identified in a number of organisms, including plants, cysts of crustaceans, rotifers (Mertens *et al.* 2008), tardigrades (Hengherr *et al.* 2009; Hinton 1951, 1960), and midges, including *B. antarctica* (Benoit *et al.* 2009a; Sakurai *et al.* 2008). However, examples such as these are infrequent, especially for polar organisms. This is partly explained by the lack of studies that have investigated the effect of desiccation on heat tolerance, but also by the fact that some organisms seem not to show cross-tolerance. For example, desiccation failed to improve heat tolerance in the bed bug, *Cimex lectularius* (Benoit *et al.* 2009b). In addition, larvae of the Antarctic midge, *E. murphyi*, failed to show greater survival at 37 and 38.5°C following 2, 4 and 8 days at 98.2% RH, and even exhibited lowered survival at 37°C following 12 days of desiccation (Everatt *et al.* in press).

Unlike the response to desiccation and low temperature stresses, some of the physiological changes that occur as a result of desiccation are different, and opposing, to those incurred as a result of high temperature. For example, the phospholipid fatty acids of the cell membrane under low temperature pack together, and transition from crystalline to gel phase, resulting in lowered membrane fluidity. Under high temperature, the phospholipid fatty acids spread apart, leading to the opposite outcome (Hazel 1995). Membrane remodelling in response to desiccation and high temperature is subsequently also antagonistic, and a prior desiccation exposure will therefore result in lowered protection. The lowered protection with regard to membrane fluidity and other processes may counteract the protection gained through physiological mechanisms that are similar between desiccation and high

temperature, such as the activation of HSPs and accumulation of polyols and sugars (Benoit *et al.* 2009a).

If this is generally the case, then cross-tolerance between desiccation and high temperature would not be expected in any organism. Yet, it does occur. It may be significant that a large proportion of the organisms that to date have shown cross-tolerance between these two stresses are anhydrobiotic (Hengherr *et al.* 2009; Hinton 1951, 1960; Mertens *et al.* 2008; Sakurai *et al.* 2008). In general, metazoan animals are only able to survive above 47°C temporarily (Addo-Bediako *et al.* 2000; Nicholas 1984; Schmidt-Nielsen 1997), and any further improvement in heat tolerance requires an animal to enter an anhydrobiotic state. This has been shown in *Polypedilum vanderplanki*, which when in anhydrobiosis was able to survive temperatures 59°C higher than when it was in its hydrated state (Hinton 1951, 1960; Sakurai *et al.* 2008). It is known that anhydrobiotic organisms have a tendency to vitrify, and also that any damage incurred as a result of denaturation, aggregation and disintegration by immobilisation ceases or slows when in this glass-like state (Crowe *et al.* 1998, 2002). These organisms may therefore not suffer the injurious changes to the membrane that would otherwise occur for non-anhydrobiotic species, and would instead only receive greater tolerance at high temperatures from being in a vitrified state. While this explanation is appropriate for anhydrobiotic organisms, the same is not true of partially desiccated organisms like *B. antarctica*, which are not vitrified when cross-tolerance is conferred to high temperatures. It is possible that the relative contribution of membrane remodelling and other opposing processes to cross tolerance is lower in these organisms. Instead, other physiological responses, including the accumulation of trehalose and the activation of HSPs (Benoit *et al.* 2009a), which are similar between desiccation and high temperature tolerance, may compensate.

In addition to cross-tolerance between desiccation and low and high temperature, cross-tolerance has also been observed between desiccation and other stresses. In particular, desiccation has been observed to improve tolerance of low-Linear Energy Transfer (LET) and high-LET radiation (Gladyshev and Meselson 2008; Gusev *et al.* 2010; Jonsson *et al.* 2008; Watanabe *et al.* 2007), CO_2 and N_2 exposure (Ricci *et al.* 2005), and high hydrostatic pressure (Seki and Toyoshima 1988). While for low temperature exposures, cross-tolerance has been reported to salinity (Elnitsky *et al.* 2009), anoxia (Yoder *et al.* 2006) and high temperature (Yoder *et al.* 2006).

6. Ecology of cross-tolerance

6.1. Ecological conditions

Although cross-tolerance has been shown to be of benefit to several polar invertebrates under laboratory conditions, there remain a number of ecological conditions that must be met for it to be successful in the field. One of these concerns the rate at which an organism is desiccated prior to or during low temperature exposure. Benoit *et al.* (2009a) discovered that faster rates of desiccation (2-13%/h) resulted in significantly less protection at -15°C in *B. antarctica*, than rates of 0.4-0.5%/h, even though percentage water loss was equivalent between treatments during low temperature exposure. It was subsequently shown that the level of trehalose was considerably lower in larvae desiccated more quickly (Benoit *et al.* 2009a). Other processes, such as the accumulation of alternative polyols and sugars, are also speculated to be affected in a similar way in *B. antarctica*. Completely desiccated or anhydrobiotic organisms are likewise affected by the rate of desiccation. Tardigrades, before entering anhydrobiosis, must first restructure their bodies into a tun-like form (Baumann 1992; Crowe 1972; Wright 1989). The formation of this tun shape is essential for the

protection of internal organs and for the reduction of water loss during anhydrobiosis (Sømme 1996). Tun formation can only take place under slow desiccation (Wright 1989).

The rate at which an organism is cooled is also important for cross-tolerance, particularly for freeze-avoiding invertebrates which utilise cryoprotective dehydration. If the SCP of these invertebrates is to remain below the ambient temperature, desiccation must proceed at a pace equivalent to that of the rate of cooling. This cannot occur if the rate of cooling is too rapid (Elnitsky *et al.* 2008b; Wharton *et al.* 2003a). The importance of a slow cooling rate is also observed during low temperature acclimation, over the long-term (Sinclair *et al.* 2003), as well as over the short-term in the form of RCH (Chidawanyika and Terblanche 2011; Kelty and Lee 1999, 2001; McDonald *et al.* 1997; Wang and Kang 2003). Rates of cooling in the Arctic and Antarctic generally tend to be slow and are sufficient to permit cryoprotective dehydration. For example, in the McMurdo Dry Valleys of continental Antarctica, natural cooling rates can average $0.021^{o}C\ min^{-1}$ (Sinclair and Sjursen 2001).

A further ecological condition that must be met by partially and severely desiccated, or cryoprotectively dehydrated, organisms, is the avoidance of inoculative freezing (= induction of freezing whilst in direct contact with ice). This is because inoculative freezing can initiate ice nucleation above an organism's SCP. For freeze-avoiding organisms this poses an inherent survival risk, while for freeze-tolerant organisms, inoculative freezing terminates desiccation and subsequently reduces the potential for cross-tolerance. Inoculative freezing is most likely to occur under moist conditions, in which ice crystals regularly pass through the cuticle and into orifices (Olsen *et al.* 1998; Salt 1963; Zachariassen and Kristiansen 2000). This increase in freezing under moist conditions has been shown in both the enchytraeid worm, *Fridericia ratzeli* (Pedersen and Holmstrup 2003), and the midge *B. antarctica* (Elnitsky *et al.* 2008). These authors therefore speculate that the microhabitat preference of some invertebrates may be influenced by the risk of inoculative freezing.

6.2. Sub-lethal characteristics

While survival is relevant to the "success" or fitness of a species, there are a number of other attributes which may be impacted upon by the sequential or simultaneous occurrence of stresses. These attributes are referred to as sub-lethal characteristics and include courtship, reproduction, foraging, predator avoidance and activity (e.g. Kelty and Lee 1999; Korenko *et al.* 2010). Because sub-lethal characteristics are affected by stresses less extreme and therefore more regularly encountered in nature than those which cause mortality, the importance of sequential or simultaneously occurring stresses may be greater than that of simple survival of extremes. However, there are only a handful of studies which have addressed this subject. Sinclair *et al.* (2007) demonstrated that *D. melanogaster* which had previously been selected for greater desiccation resistance/tolerance had a shorter, and thus improved, chill coma recovery time as compared with controls. Everatt *et al.* (2013) showed that the critical thermal minimum (CTmin, the low temperature at which an organism no longer shows coordinated movement) and chill coma (low temperature at which electrophysiological activity ceases completely) of *C. antarcticus* were negatively affected by prior desiccation on concentrated sea water. However, due to the limited number of such studies, and that they have focussed only on the thermal thresholds of activity to date, it is not yet possible to draw conclusions on the effect of sequential and simultaneous stresses on sub-lethal characteristics.

6.3. Climate warming

Over the last two to three decades, climate warming has received considerable public attention and has become the focus of the largest scientific collaboration in human history. There is now an almost

universal consensus that atmospheric CO_2 levels are rising as a result of human activity and are leading to warming on a global scale. Temperatures have so far risen, on average, by 0.85°C across the Earth's surface over the last century (IPCC 2013). The rate of increase in temperature has been particularly high in certain polar regions ('polar amplification'), averaging 2°C over the past 50 years (Arctic Council 2005; Convey *et al.* 2009; Turner *et al.* 2009). Furthermore, general circulation models suggest these temperature trends will continue (Convey *et al.* 2009; Turner *et al.* 2009). Water availability is also likely to change as a result of climate warming. Precipitation is predicted to increase by 0.5-1% per decade at higher latitudes (Walther *et al.* 2002; Turner *et al*. 2009). However, as temperatures rise, snow cover is expected to decrease and melt is expected to occur earlier in the season. In turn, the thawing of glaciers and evaporation of meltwater are also expected to take place earlier in the summer season (Avila-Jimenez *et al.* 2010; Walther *et al.* 2002).

Polar terrestrial invertebrates will therefore increasingly experience warmer and drier summers. Understanding the capability of these invertebrates to tolerate high temperature and low water availability either sequentially or simultaneously thus becomes increasingly significant. As discussed above, the level of cross-tolerance between high temperature and low water availability is variable. While invertebrates like the midge, *B. antarctica*, are afforded greater heat tolerance following prior desiccation, others including the closely related midge, *E. murphyi*, and the collembolan, *C. antarcticus*, are incapacitated once desiccated (Benoit *et al.* 2009a; Everatt *et al.* 2013,in press). The success of polar invertebrates in a warming climate will therefore be species specific. However, recent studies have demonstrated that some of these invertebrates possess considerable basal heat tolerance over both short and long timescales, and the heat tolerance shown far exceeds that required during current and foreseeable Antarctic and Arctic summers (Deere *et al.* 2006; Everatt *et al.* 2013, in press; Sinclair *et al.* 2006; Slabber *et al.* 2007). The Collembola, *C. antarcticus* and *M. arctica*, for example, are able to survive above 30°C for a number of hours and at 10°C for over 40 days (Block *et al.*, 1994; Hodkinson *et al.*, 1996; Everatt *et al.* 2013, in press). The level of tolerance these invertebrates show is in line with the thermal sensitivity hypothesis, that the sensitivity of terrestrial invertebrates to higher temperature exposure decreases with increasing latitude (Addo-Bediako *et al.* 2000; Deutsch *et al.* 2008).

7. Conclusions and future directions

Many polar terrestrial invertebrates are ancient and have likely spent the last few million years honing their physiology to the extreme environments in which they live (Convey and Stevens 2007; Marshall 1996; McGaughran *et al.* 2010). The tolerance these animals have to sub-zero temperatures and desiccation stress has been known for many decades, yet it is only relatively recently that investigations into the molecular mechanisms underpinning these physiological adaptations have begun. Molecular responses to sub-lethal chilling also remain poorly characterised, and disentangling the processes underpinning chill vs. extreme cold or freezing damage is an important future challenge. A possible route to addressing this would be to undertake more detailed time series investigations of the stress response cascade to declining temperature. This includes key functional, i.e. not just tolerance, phenotypes such as activity thresholds and metabolic adaptation as set out in Fig. 1. Crucially, future studies need to more directly address mechanism, rather than simple correlations between molecular and phenotypic changes that currently dominate the literature. This extends to understanding responses to multiple stressors in tandem, which is another current knowledge gap. The protection afforded against one stress following exposure to another was no doubt pivotal in the persistence of many species during historic climate transitions, as well as important currently during winter in the polar regions. This is perhaps most evident in animals which cryoprotectively dehydrate

like *M. arctica*, whose sole strategy of surviving winter relies on cross-tolerance to cold and desiccation.

A benefit of cross-tolerance between high temperature and desiccation has received even less attention, but is a combination of stressors which may occur more frequently in polar environments under continued climate warming. While some species show cross-tolerance, others do not, and further work is required to establish a baseline for different species groups, as well as for polar terrestrial invertebrates generally. However, it should be noted that many of these invertebrates show a level of heat tolerance that is far greater than is required for even medium term predictions of climate warming. Thus, physiological approaches must be guided by more detailed studies of current microclimate conditions, and models forecasting rates of environmental change, to better predict winners and losers under different climate scenarios. A greater threat to survival may in fact be competition from newly colonising species – and investigating the physiological 'suitability' of species whose distribution boundaries place them on the doorstep of polar environments is another important research objective (Everatt *et al.* 2012; Frenot *et al.* 2005).

To end, this review has shown the almost boundless flexibility and adaptability enlaced into the physiology of invertebrates, when faced with a single stressor or multiple stressors. Such is their adaptability that they have been able to thrive in many habitats, even those as extreme and inhospitable as the Arctic and Antarctic. The review has also highlighted the importance of looking at all factors together and not simply investigating single factors in isolation. As the field of invertebrate stress ecophysiology grows, so will the need for a cross-disciplinary approach.

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Highlights

- Invertebrates are vulnerable to extremes of temperature and low water availability.

- They express a suite of features in response to each stressor individually.

- However, stresses usually occur simultaneously in nature.

- Cross-tolerance is observed between temperature and water stress.

- This is discussed in light of climate warming and the invertebrates' ecology.

Figure Captions

Figure 1 Schematic representation of the molecular and physiological processes underpinning chilling, Freeze Tolerance (FT) and Freeze Avoidance (FA) strategies in terrestrial invertebrates. Chilling/cold stress responses can be induced in parallel or more sequentially - the order in which temperature induces each mechanism will be species specific and potentially vary depending on the rate of temperature change. Common chilling responses (light grey) are shared by both FT and FA strategies to limit chilling injury. Fundamental differences between FT (white) and FA (dark grey) include the synthesis of ice nucleating agents (INAs) in FT (white) *vs.* INA removal in FA. FT insects also uniquely produce osmoprotectants to control cellular dehydration during extracellular freezing. Both strategies employ cryoprotectants (e.g. glycerol and trehalose) and antifreeze proteins (AFPs); and can potentially undergo vitrification. For FT species these strategies facilitate controlled freezing and limiting freezing damage, while in FA species these adaptations enhance the supercooled state/reduce the risk of ice-crystal formation. See main text for details of relevant studies. Adapted from Bale (2002).

1484
1485 **Figure 2** Schematic representation of the molecular and physiological processes underpinning
1486 Desiccation Tolerance (DT) and Desiccation Resistance (DR). The three categories of desiccation
1487 resistance are Mesic (highly resistant to water loss), Transitional (intermediate resistance) and Hygric
1488 (limited desiccation resistance). At polar latitudes in particular, limited desiccation resistance
1489 necessitates increased desiccation tolerance. The purpose of DR mechanisms (white) is to reduce
1490 water loss. The purpose of DT mechanisms (dark grey) is to limit desiccation-induced damage.
1491 Some strategies are used by both DR and DT (light grey). See main text for further details.
1492